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Effect of Anaerobiosis on Staphylococcal Nuclease Production

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Five strains of *Staphylococcus aureus* were examined quantitatively for the production of nuclease under aerobic and anaerobic conditions. Hydrolysis of deoxyribonucleic acid and ribonucleic acid was detected by measuring the release of acid-soluble nucleotides spectrophotometrically. We found that the enzyme was produced anaerobically, as well as aerobically, and that anaerobiosis had no effect on production of this enzyme when other conditions, such as *p*H, were held constant.

Since the discovery (5) that *Staphylococcus aureus* produces an extracellular nuclease, considerable study has been done on the chemistry of the isolated enzyme (1, 2). Cunningham et al. (5) found that staphylococcal nuclease differed from pancreatic deoxyribonuclease in that it split the 5′ bond leaving fragments with terminal phosphate groups at the 3′ position and in that it required calcium for activity. A later investigation (10) indicated that the optimal *p*H for enzyme production was on the alkaline side of neutrality, and the optimal *p*H for activity was reported to be about 10.3 (1). Both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were hydrolyzed by the purified enzyme (2, 7). Nuclease production was found by Weckman and Catlin (10) to parallel growth. It is first detectable in early log phase and reaches a maximum during early maximal stationary phase.

While investigating over 300 strains of staphylococci isolated from hospital patients, Di Salvo (6) found a 100% correlation between the production of coagulase and deoxyribonuclease by these organisms. Burns and Holtman (3, 4) discovered that the vast majority of the strains of *S. aureus* they isolated from a hospital environment produced deoxyribonuclease and coagulase. They also observed that the strains isolated from typical staphylococcal lesions were more active biochemically than strains isolated from less typical sources, such as sputum, blood, and urine. During the course of their experiments, it was also noted that *S. aureus* could hydrolyze DNA under aerobic or anaerobic conditions, but that RNA was hydrolyzed only in a state of aerobiosis. Other investigators in our laboratory later observed that both substrates were hydrolyzed by *S. aureus* under anaerobic conditions.

To be an effective weapon in the pathogenicity of staphylococci, an enzyme would apparently have to be produced during growth under reduced oxygen tension because staphylococcal lesions are considered essentially anaerobic. Because the literature is controversial as to whether staphylococcal nuclease is produced under these conditions, we attempted to determine, by quantitative methods, the effect of anaerobiosis on nuclease production by staphylococci.

MATERIALS AND METHODS

Microorganisms. The organisms used were obtained from stock cultures at The University of Tennessee or were isolated from pathological material at Fort Sanders Hospital in Knoxville, Tenn. The physiological characteristics of the organisms were determined by standard laboratory techniques and were found to conform to the species definition of *S. aureus.*

Media. The medium for quantitative determination of nuclease production consisted of Brain Heart Infusion (BHI; Difco) to which 8.0 g of glucose and 42.0 mg of $NaHCO_3$ were added per liter. The medium was dispensed into 2-liter flasks. Amounts of 1 liter of the liquid were added to the flasks for aerobic growth, and 1.5-liter amounts were added for anaerobic growth. The flasks for anaerobic culture were sealed with rubber stoppers at the time of sterilization to prevent absorption of air.

Growth conditions. Cultures (24 hr old) of the stock organisms were used. Each was washed once, and the absorption of the suspensions was adjusted to 1.0 on a Bausch & Lomb Spectronic-20 colorimeter at 600 mμ. Each suspension (1 ml) was used to inoculate 1 liter of medium.

Cultures were grown aerobically and anaerobically for 18 hr at 35 C. At this time the cultures were in early maximal stationary phase. Aeration was accomplished by pumping air into the medium through a

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candle-type porcelain filter. During growth the medium was maintained at *p*H 7.4 by means of a Radiometer automatic titrator, model TTT1, with a magnetic relay (MRC-1) and an adaptor for Beckman calomel glass electrodes. NaOH (1 M) was titrated into the medium by a stainless-steel solenoid valve attached to a reservoir.

Quantitation of growth. The cell yield was expressed as the absorbance of the culture. Samples of each culture were diluted with 0.15 M NaCl so that the optical density (OD) was 0.6 or less. The OD values thus obtained were directly proportional to cell concentration. The resulting OD_{600} was multiplied by the dilution factor to obtain the absorbance of the culture.

Assay of nuclease activity. A sample of each culture was centrifuged, and the supernatant liquid was assayed by a modification of the procedure reported by Alexander et al. (1). The substrates were thymus DNA and yeast RNA in concentrations of 1.0 mg/ml in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.6. The buffer was made 0.01 M with respect to $CaCl_2$. An amount of 1 ml of substrate and 0.1 ml of culture supernatant fluid were incubated at 37 C for 20 min in the deoxyribonuclease assays and for 30 min in the evaluation of ribonuclease. After incubation, 0.5 ml of 10% perchloric acid was added, and the tubes were cooled in an ice bath for 15 min. The broth-perchloric acid mixtures were diluted with 2.0 ml of water, and the acid-insoluble nucleic acid was sedimented by centrifugation. The extent of hydrolysis was determined by measuring the absorption on the acid-soluble nucleotides spectrophotometrically at 260 mμ. A control for each test consisted of adding the culture supernatant liquid after the introduction of perchloric acid. The OD of this control tube was subtracted from the OD of the tube under test, and the resulting difference was then used to calculate the enzyme concentration from the standard curve. The perchloric acid used as the RNA precipitant contained 0.25% uranyl acetate.

A standard curve was made for each substrate with purified staphylococcal nuclease, chromatographically pure (Worthington Biochemical Corp., Freehold, N. J.), diluted in Tris buffer.

Statistical analysis. To determine whether there was any significant difference between aerobic and anaerobic production of nuclease, *t* was calculated for the results of the assays for deoxyribonuclease and ribonuclease by Snedecor's method (9).

RESULTS

Effect of anaerobiosis on growth of staphylococci. Although the staphylococci used in this study grow anaerobically, the total cell yield under anaerobic conditions was much less than under aerobic conditions. Only about one-fourth as much cell yield was obtained anaerobically as was obtained aerobically (Table 1).

Production of nuclease under aerobic and anaerobic conditions. For determination of the effect of anaerobiosis on nuclease production, each test organism was assayed for the production of enzyme after growth for 18 hr in both the presence and the absence of air. Preliminary experiments had shown that under the conditions employed the cultures were in maximal stationary phase at 14 to 16 hr. The enzyme was produced under either aerobic or anaerobic conditions as determined on both RNA and DNA (Table 2).

TABLE 1. *Effect of anaerobiosis on growth*

Organism	Optical density (600 mμ)	
	Aerobically grown	Anaerobically grown
UT46	5.850	1.720
FS8	8.325	1.175
FS10	7.095	1.450
FS13	6.930	1.525
FS16	8.250	2.350

TABLE 2. *Effect of anaerobiosis on staphylococcal nuclease production*

Organism	Substrate			
	DNA		RNA	
	Enzyme concn	Specific activity[a]	Enzyme concn	Specific activity[a]
	mμg/ml		*mμg/ml*	
UT46				
Aerobic..	6,560	1,290	7,920	1,354
Anaerobic.....	1,635	951	1,800	1,047
FS8				
Aerobic..	4,160	500	4,400	529
Anaerobic	750	638	330	281
FS10				
Aerobic..	1,940	273	1,540	217
Anaerobic	195	134	237	163
FS13				
Aerobic..	2,260	326	2,560	369
Anaerobic	885	580	345	226
FS16				
Aerobic..	1,640	199	2,280	276
Anaerobic	270	115	339	144

[a] Millimicrograms of enzyme/optical density of culture.

The total amount of enzyme produced under aerobic conditions was much greater than that produced anaerobically. However, when the specific activity was calculated by dividing the total enzyme production of each culture by the cell yield, it was obvious that there was little difference in the amount of enzyme produced per cell.

The different strains of *S. aureus* used in this study varied considerably with regard to the amount of enzyme produced under the conditions employed. Aerobically *S. aureus* UT46 had spe-

cific activities of 1,290 on DNA and 1,354 on RNA, whereas *S. aureus* FS10 was much less active with specific activities of 273 and 217 on DNA and RNA, respectively.

Statistical analysis. The *t* distribution was applied to the specific activities of the aerobic and anaerobic culture of each organism, and a value of *t* was obtained for RNA and DNA. When RNA was used as the substrate, *t* was calculated to be 0.658. With DNA as the substrate *t* was found to be 0.095. The critical value of *t* at the 95% confidence level is 2.776 (9). Since the values of *t* for both ribonuclease and deoxyribonuclease are less than the critical value, it can be concluded that there is no significant difference in the amount of enzyme produced under aerobic and anaerobic conditions for either substrate at the 95% confidence level.

DISCUSSION

All of the strains of *S. aureus* tested were found to produce ribonuclease and deoxyribonuclease. There is considerable variation between the strains in the relative amounts of ribonuclease and deoxyribonuclease activity, and the ratio, ribonuclease/deoxyribonuclease, varies from strain to strain. This ratio for *S. aureus* FS8 is 1.06 and for *S. aureus* FS10 is 0.79 under aerobic conditions.

The cell yield anaerobically was only about one-fourth of that obtained aerobically, and the total amount of enzyme produced under this condition was proportionally lower than that produced aerobically. Statistical analysis of the data indicated that anaerobiosis had no effect on the amount of nuclease produced per cell if other conditions, such as *p*H, were held constant. The same observation was also made when the organisms were tested by the qualitative streak-plate method. This could explain why Burns and Holtman (3) and M. R. Cartledge (M.S. Thesis, Univ. of Tennessee, Knoxville, 1963) were unable to detect ribonuclease activity anaerobically when the streak-plate procedure was employed. The qualitative determination for ribonuclease was found to be slightly less sensitive than the test for deoxyribonuclease.

The results suggest that qualitative methods are not entirely satisfactory for detecting an enzyme system under conditions which are known to affect growth. In the qualitative detection of staphylococcal nuclease, anaerobiosis affects the final *p*H of the medium. Aerobically, the *p*H rises to about 8.0, whereas anaerobically a final *p*H of about 5.5 is attained. Since the optimal *p*H for enzyme activity is about 9.0, anaerobic conditions would tend to reduce the sensitivity of the test procedure. The addition of mannitol to the DNA agar is not recommended because of the acid produced by mannitol-fermenting strains.

Other investigators have shown that inflammatory exudates contain large amounts of nucleoprotein (8). It is possible that staphylococcal nuclease could provide the organism with considerable amounts of low molecular weight nutrients from this source. Uracil, which is required by staphylococci for anaerobic growth, might be made available to the organism by such a mechanism. Also, staphylococcal nuclease has been found to be leukocidal and is not neutralized by specific antibody (G. M. Nickerson and W. R. Chesbro, Bacteriol. Proc. p. 77, 1963). It is possible that the enzyme may function in the formation of staphylococcal lesions by protecting the bacterial cell from phagocytosis.

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